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(54) **Monoclonal antibody recognizing membrane phospholipase A2 and immunoassay of membrane phospholipase A2.**

(57) The present invention first provides monoclonal antibodies recognizing membrane phospholipase A_2, namely, monoclonal antibodies PL-49, PL-71, PL-76, and PL-78, hybridomas producing them, methods for producing them, and immunoassays of membrane phospholipase A_2 using them.

The immunoassay of PLA_2M is useful for the diagnosis of articular rheumatism, cancers, and a wide variety of inflammatory states.

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The present invention relates to monoclonal antibodies recognizing membrane phospholipase A_2; hybridomas producing said monoclonal antibodies; a method for producing said monoclonal antibodies; and immunoassays using said monoclonal antibodies.

Phospholipase A_2 (PLA_2) (EC 3.1.1.4) is an enzyme which can hydrolyze the fatty acyl ester bond at the sn-2 position of glycerophospholipids. It is well known that the enzyme is present in the pancreas or snake venom, and it has been observed that the level of pancreatic PLA_2 in blood increases in patients suffering from pancreatitis (Ogawa et al., Gendai Iryou, 20 (1988) 3013-3017). The PLA_2, however, is not only present in the external secretion system, but also found in almost all of the cells in a living body, although the amount thereof is very small (Van den Bosch, H. in Phospholipids (Hawthrone, J. N. and Ansell, G. B. eds.) (1982) pp. 313-357, Elsevier/North-Holland Biomedical Press, Amsterdam). It is believed that the enzyme would play an important role in the metabolic regulation of membrane phospholipids and in the eicosanoide biosynthesis through arachidonic acid (Van den Bosch, H., Biochim. Biophys. Acta (1980) 604, 191-246), and would relate to inflammation and cellular injury through the direct action or through its metabolites such as lysophospholipids, leukotrienes, platelet-activating factor and lipid peroxides (Vades, P. et al., Lab Invest. (1986) 55, 391-404).

By analysis of the protein primary structure it was revealed that membrane PLA_2 (PLA_2M) isolated from human splenic membrane fraction was a type of PLA_2 which is different from pancreatic PLA_2 - (Kanda, A. et al., Biochem. Biophys. Res. Commun. (1989) 163, 42-48), and it was also found that PLA_2M was induced by an inflammatory mediator such as IL-1 and TNF and secreted out of the cells (Nakano, T. et al., FEBS Lett. (1990) 261, 171-174). Moreover, a comparison between PLA_2M and PLA_2 purified from rheumatoid arthritic synovial fluid showed that they are identical in their structure and reactivity (Kramer, R. M. et al., J. Biol. Chem. (1989) 264, 5768-5775).

From a clinical point of view, an increase of the PLA_2 enzymatic activity was found in the blood of patients with an infectious disease such as septicemia, pustular psoriasis, Crohn's disease, and rheumatoid arthritis. Further, it was found that the PLA_2 enzymatic activity is induced by intracutaneous injection of bacteria, viruses, or other inflammatory inducers into an animal (Vades, P. et al., supra).

To date, however, no report has appeared concerning the assay of PLA_2M, and it has not yet been shown whether an increase of the PLA_2 enzymatic activity accompanying the aforesaid diseases is caused by PLA_2M or not.

As described above, because the PLA_2 enzymatic activity in blood is increased when suffering from rheumatism, septicemia, pustular psoriasis, Crohn's disease, or the like, it has been expected that the diagnosis of these diseases can be realized from the measurement of PLA_2M and an assay of PLA_2M has been desired. Therefore, it is the object of the present invention to provide monoclonal antibodies which can be used in an immunoassay of membrane phospholipase A_2.

This object is achieved by the provision of monoclonal antibodies recognizing membrane phospholipase A_2, namely, monoclonal antibodies PL-49, PL-71, PL-76, and PL-78. The membrane phospholipase A_2 is preferably derived from human spleen. The present invention further provides hybridomas producing the monoclonal antibodies. The monoclonal antibodies can be produced by growing the hybridomas in the abdominal cavity of a mouse and separating the monoclonal antibodies from the ascitic fluid accumulated in the abdominal cavity. The present invention also provides an immunoassay of membrane phospholipase A_2 using the monoclonal antibodies. The preferred immunoassay is a radioimmunoassay. In a sandwich immunoassay, an enzyme immunoassay is preferred.

The immunoassay of PLA_2M using the monoclonal antibodies is useful not only for the diagnosis of articular rheumatism but also for the diagnosis of cancers and a wide variety of inflammatory states including an external wound.

Brief Description of the Drawings

Figure 1 shows a calibration curve for PLA_2M in the sandwich assay using PL-78 and PL-71-peroxidase conjugate.

Figure 2 shows a chromatogram obtained in the gel filtration of ^{125}I-labeled solution of PLA_2M.

Figure 3 shows a standard curve in the RIA of PLA_2M.

Figure 4 shows a dilution curve for human sera.

Figure 5 shows the results of ion exchange chromatography of the standard PLA_2M and sera from patients with articular rheumatism.

Figure 6 shows the concentrations of PLA_2M in sera from normal individuals, patients with articular rheumatism, patients with cancer, and patients with an external wound.

The present invention provides monoclonal antibodies recognizing membrane phospholipase A_2. The membrane phospholipase A_2 is preferably derived from human spleen, as described in Biochem. Biophys. Res. Commun. Vol. 163, No. 1, 1989, pp. 42-48. In the present invention, monoclonal antibody PL-49, monoclonal antibody PL-

71, monoclonal antibody PL-76, and monoclonal antibody PL-78 were obtained as detailed in Examples below.

The present invention further provides hybridomas producing the corresponding monoclonal antibodies described above. The hybridoma PL-49, hybridoma PL-71, hybridoma PL-76, and hybridoma PL-78 can produce monoclonal antibody PL-49, monoclonal antibody PL-71, monoclonal antibody PL-76, and monoclonal antibody PL-78, respectively. These hybridoma PL-49, hybridoma PL-71, hybridoma PL-76, and hybridoma PL-78 were deposited on May 9, 1990, under the terms of the Budapest Treaty with Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305 Japan, and have been assigned Mouse hybridoma PL-49 with the Accession No. FERM BP-2891, Mouse hybridoma PL-71 with the Accession No. FERM BP-2892, Mouse hybridoma PL-76 with the Accession No. FERM BP-2889, and Mouse hybridoma PL-78 with the Accession No. FERM BP-2890, respectively.

The aforesaid monoclonal antibodies can be produced by growing the corresponding hybridomas described above in the abdominal cavity of a mouse, and separating the monoclonal antibodies from the ascitic fluid accumulated in the abdominal cavity.

The present invention also provides an immunoassay of membrane phospholipase A_2 using the aforementioned monoclonal antibodies. The preferred immunoassay is a radioimmunoassay. In the immunoassay where the membrane phospholipase A_2 is sandwiched between two different species of the aforesaid monoclonal antibodies, an enzyme immunoassay is preferred.

Example 1

Preparation of monoclonal antibodies against membrane PLA_2 (PLA_2M)

(1) Immunization

The method for preparing PLA_2M used in the immunization and the assay of antibodies was as described in Biochem. Biophys. Res. Commun. Vol. 163, No. 1, 1989, pp. 42-48.

First immunization: a solution of PLA_2M in phosphate-buffered saline (PBS) was mixed with Freund's Complete Adjuvant (FCA) to form an emulsion in a ratio of 1:1. The emulsion was administered subcutaneously to eight mice (Balb/C, female, 12 weeks old) at a dose equivalent to 2 µg of protein for each mouse.

Second immunization: It was carried out 23 days after the first immunization in the same manner as that of the first immunization.

Third immunization: it was carried out 44 days after the second immunization in the same manner as that of the first immunization.

Fourth immunization: it was carried out 81 days after the third immunization in the same manner as that of the first immunization.

(2) Determination of serum titer

The level of anti-PLA_2M antibody in the blood of the immunized animals was determined by ELISA. That is, 0.5 ng of PLA_2M in 0.1 ml of 0.1 M $NaHCO_3$ was added to each well of a microtiter plate and left overnight at 4°C to coat the plate, after which 0.3 ml of 1% bovine serum albumin (BSA) in PBS was added thereto and the mixture was incubated at 37°C for 1 hour to block the plate. Then, 0.05 ml of a sample was added and allowed to react at 37°C for 1 hour, followed by an assay with a Vectastain ABC kit (mouse IgG kit, Vector Laboratories, Inc.) according to its protocol. As a color former, 1 mg/ml of orthophenylenediamine (OPD) was used and the difference in absorbances at 492 nm and at 660 nm was determined by a Corona microplate photometer MTP-22. The mice exhibiting a high PLA_2M antibody titer were used for hybridoma preparation.

(3) Preparation of hybridomas

(3.1) Experiment 1

Thirty-one days after the fourth immunization, a fifth immunization was carried out. Four micrograms of PLA_2M were dissolved in 0.2 ml of PBS and the resulting solution was administered intraperitoneally to the mice. Three days after the fifth immunization, a cell fusion was carried out. The method for the cell fusion was substantially the same as that of Galfre and Milstein (Methods Enzymol. 73, 46 (1981)). That is, mouse myeloma cells (P3X63-Ag8.653) were cultured in an RPMI1640 medium (90% RPMI1640; 10% fetal calf serum; 0.15 mg/ml sodium pyruvate; 0.15 mg/ml oxaloacetic acid; and 0.1 mg/ml kanamycin). From the resulting culture, 4.67×10^7 mouse myeloma cells were harvested and mixed with 9.33×10^7 spleen cells of the immunized mice, and the combined cells were pelleted by centrifugation in a centrifugation tube and 1 ml of 48% polyethylene glycol 4000 solution was added dropwise over 1 minute. The mixture was then stirred for 1.5 minutes, followed by slow addition, in a dropwise fashion with stirring, of a serum-free RPMI1640 medium in an amount of 2 ml over 2 minutes, 2 ml over 1 minute, and 6 ml over 2 minutes. Finally, 15 ml of the same medium was gently added and the

mixture was pelleted by centrifugation. The pellets obtained were suspended in an HAT medium (70% RPMI1640; 10% NCTC109; 20% fetal calf serum; 10^{-4}M hypoxanthine; 4 x 10^{-7}M aminopterin; 1.6 x 10^{-5}M thymidine; 0.15 mg/ml sodium pyruvate; 0.15 mg/ml oxaloacetic acid; 0.2 IU/ml insulin; 2.5 x 10^{-4}M 2-mercaptoethanol; 5 x 10^{-3}M HEPES; 0.1 mg/ml kanamycin; and nonessential amino acids). The suspension was adjusted to have a concentration of 0.75 x 10^6 spleen cells per milliliter and then dispensed as 0.2 ml portions into each well of 96-well plates. The plates were incubated in 95% air-5% CO_2 at a temperature of 37°C and humidity of 95% or more, and the medium was replaced, if necessary, half by half with a fresh HAT medium.

(3.2) Experiment 2

Thirty-five days after the fourth immunization, a fifth immunization was carried out in the same manner as in Experiment 1. Three days after the fifth immunization, a cell fusion was carried out. As the mouse myeloma cell strain, 8.89 x 10^7 P3X63-Ag.8.653 cells were used for the cell fusion with 1.7 x 10^8 mouse spleen cells in the same manner as in Experiment 1. The cells subjected to the cell fusion were suspensed in an HAT medium to have a concentration of 0.83 x 10^6 cells/ml, and the suspension was then dispensed as 0.2 ml portions into each well of 96-well plates, followed by incubation in the same manner as in Experiment 1.

(4) Screening of hybridomas

After about 2 weeks the culture supernatant of hybridomas which grew was examined whether anti-PLA_2M antibodies were produced therein or not. The assay was carried out in the same manner as that of Item (2). Four hybridomas (PL-49, PL-71, PL-76 and PL-78) each of which stably produced an antibody having a specific reactivity with PLA_2M were obtained by the screening.

(5) Cloning of hybridomas and storage in frozen state

The aforesaid four hybridoma cells were cloned by a limiting dilution technique. That is, each of the hybridomas was suspended in an RPMI1640 medium and the suspension was added to a 96-well plate to have a concentration of 0.3 cells in 0.2 ml for each well, followed by incubation. The anti-PLA_2M antibody titer of the culture supernatant was determined in the same manner as that of Item (4). The anti-PLA_2M antibody-producing hybridomas were selected and grown, after which they were stored under freezing in a freezing solution (90% fetal calf serum and 10% dimethylsulfoxide).

(6) Preparation of ascitic fluid

To each group of mice (Balb/C, female, 10-15 weeks old) to which 0.5 ml of pristane had been administered intraperitoneally 7-10 days earlier, a suspension of each of the hybridoma cells in PBS (2-5 x 10^6 cells/ml) was administered intraperitoneally at a dose of 0.5 ml for each mouse. After about 1 week, an ascitic fluid accumulated in the respective mice which was collected by a tapping technique. From the ascitic fluid collected, precipitate was removed by centrifugation using a Separapid tube (SEKISUI KAGAKU). The ascitic fluid so treated was dispensed and stored under freezing.

(7) Determination of antibody class and subclass

The immunoglobulin class and subclass of the monoclonal antibodies produced by the respective hybridomas were determined by ELISA. A mouse MonoAb-ID-EIA kit (Zymed Co., Ltd.) was used for the determination. In all cases of the four hybridomas, PL-49, PL-71, PL-76 and PL-78, immunoglobulins produced thereby were identified as IgG_1 (γ1, κ).

(8) Purification of antibodies

The monoclonal antibodies were purified from the ascitic fluid by the use of an Affigel protein A MAPS II kit (Bio-Rad Co., Ltd.) according to its protocol.

(9) Preparation of peroxidase conjugate

According to the method of Nakane et al. (J. Histochem. Cytochem., 22, 1084 (1974)), a conjugate of PL-71 with horseradish peroxidase was prepared. First, 0.1 ml of 0.1 M $NaIO_4$ was mixed with peroxidase (2 mg/0.5 ml in water) and the mixture was allowed to react at room temperature for 20 minutes. The reaction mixture was dialyzed overnight at 4°C against 1 mM sodium acetate buffer and then adjusted to pH 9.5 with 0.2 M Na_2CO_3, followed by addition of PL-71 (4 mg/ml in 0.01 M $NaHCO_3$). The mixture was then stirred at room temperature for 2 hours and 50 μl of $NaBH_4$ - (4 mg/ml in water) was added thereto. The mixture was also stirred at 4°C for 2 hours and dialyzed overnight at 4°C against PBS to give the desired conjugate.

(10) Sandwich assay of PLA_2M

A solution of monoclonal antibody (PL-78) was

added to each well of a microtiter plate (at a concentration of 0.1 μg in 0.1 ml of $NaHCO_3$ for each well). After standing overnight at 4°C, 0.3 ml of PBS containing 1% BSA was added to the plate, which was then incubated at 37°C for 1 hour, thereby blocking the plate. Thereafter, 0.05 ml of PLA_2M solution (1% BSA in PBS) was added to the plate and allowed to react at 37°C for 1 hour. Then, 0.05 ml of an 1,000-fold dilution of the peroxidase conjugate with monoclonal antibody PL-71 was added to the plate and allowed to react at 37°C for 2 hours. Thereafter, orthophenylenediamine (at a concentration of 1 mg in 0.1 ml of 0.1M citrate buffer, pH 4.2) and H_2O_2 (at a final concentration of 0.03%) were added to the plate and allowed to react at room temperature for 30 minutes. Finally, the reaction was stopped by adding 0.1 ml of 1 N H_2SO_4 and the difference in absorbances at 492 nm and at 660 nm was measured. Figure 1 shows a calibration curve of PLA_2M in the sandwich assay using PL-78 and the peroxidase conjugate with PL-71. The sensitivity for detecting PLA_2M by the present method was approximately 0.01 ng/well (see Figure 1).

Example 2

Radioimmunoassay (RIA) of membrane phospholipase A_2 (PLA_2M)

(1) Preparation of ^{125}I-labeled PLA_2M

The ^{125}I-labeled PLA_2M was obtained by a chloramine T technique in accordance with the Hunter-Greenwood method (Nature, 194, 495-496 (1962)).

① Reagents

PLA_2M solution:
111 μg/ml in 0.5 M phosphate buffer (PB) (pH 7.4),
$Na^{125}I$ solution:
3.7 GBq/ml in dilute NaOH solution (pH 7-11)
Chloramine T solution;
2 mg/ml in 0.5 M PB (pH 7.4),
Sodium pyrosulfite solution:
2.5 mg/ml in 0.1 M PB (pH 7.4),
BSA:
10 mg/ml in 0.1 M PB (pH 7.4)

② Procedure

First, 25 μl of 0.5 M PB (pH 7.4) and 22.5 μl of the PLA_2M solution were put in a polypropylene tube, after which 2.5 μl of the $Na^{125}I$ solution were added and the contents were mixed well together. Then, 2.5 μl of the chloramine T solution were also added and the contents were stirred at room temperature for 50 seconds. To this tube, 12.5 μl of the sodium pyrosulfite solution were further added and the contents were stirred, after which 2.5 μl of BSA and 2.5 μl of potassium iodide solution were added and the contents were mixed well together. The resulting mixture was then subjected to gel filtration (the column was PD-10 (manufactured by Pharmacia Co., Ltd.); the eluent was 0.1 M PB (pH 7.5) containing 0.5M sodium chloride, 0.5% BSA, and 0.05% sodium azide), and 1 ml-fractions of the eluate were collected. The radioactivity of each fraction was measured by means of a well-type scintillation counter. The chromatograms obtained are shown in Figure 2. Fraction No. 4 in this figure was identified as ^{125}I-labeled PLA_2M.

(2) RIA of PLA_2M

① Reagents

PLA_2M standard solution: 0.2 - 200 ng/ml assay buffer,
Ascitic dilutions: 680,000-, 470,000-, 860,000- and 2,300,000-fold dilutions of PL-49, PL-71, PL-76 and PL-78,
respectively, with the assay buffer,
^{125}I-labeled PLA_2M solution: a dilution of the labeled solution as described in Item (1) with the assay buffer (2×10^6 cpm/ml),
Immunobead liquid: a suspension of rabbit anti-mouse immunoglobulin-bound polyacrylamide gel (manufactured by Bio-Rad Co., Ltd.) in the assay buffer (1 mg/ml),
Assay buffer: 0.1 M PB (pH 7.5) containing 0.5 M sodium chloride, 1 mM ethylenediaminetetraacetic acid, 0.5% BSA, and 0.02% sodium azide.

② Procedure

First, 100 μl of the PLA_2M standard solution or a serum sample were put in a polypropylene tube, after which 275 μl of the assay buffer and 25 μl of the ^{125}I-labeled PLA_2M solution were added and the contents were mixed well together. Then, 100 μl of the ascitic fluid dilution were also added and the contents were incubated at room temperature for 16 hours. To this tube 100 μl of the immunobead liquid were further added and the contents were incubated at room temperature for 1 hour, followed by centrifugation (2000 x g, 10 min). After the removal of the supernatant by suction, the radioactivity of the precipitate was measured by means of a well-type scintillation counter. Based on the readings from a standard curve obtained by the use of the PLA_2M standard solution, the concentration of PLA_2M in the serum sample was determined.

③ Standard curve and sensitivity

Figure 3 shows a standard curve obtained in the RIA when PL-49 was used as an ascitic fluid. Other ascitic fluids gave similar competition curves. The sensitivity (i.e., concentration for 90% inhibition) was 0.3 ng/ml for PL-49, 1.1 ng/ml for PL-71, 0.9 ng/ml for PL-76 and 0.2 ng/ml for PL-78, all of which were considered to be highly sensitive.

④ Specificity

As shown in Figure 4, all the dilution curves for human sera obtained by the present method exhibited a good liner regression and there appeared no influence of sera on the curves.

Moreover, no cross reaction of human pancreas PLA_2 was observed in the present method.

In addition, standard PLA_2M and sera from patients with articular rheumatism were subjected to ion exchange chromatography (column was S-Sepharose Fast Flow Type (manufactured by Pharmacia Co., Ltd.); eluent was 50 mM Tris buffer (pH 7.0) with a linear gradient containing from 0.2 to 1M sodium chloride and 0.1% CHAPS (manufactured by Dotite Co., Ltd.)). The eluate was fractionated and PLA_2M present in each fraction was measured by the present method. The results provided a chromatogram as shown in Figure 5, indicating that the pattern of the chromatogram obtained by the standard PLA_2M had a good fit to that obtained by the sera of patients.

These results revealed that the present method is applicable to a specific assay of PLA_2M present in human sera.

⑤ Assay of human sera

According to the present method, various sera from normal individuals, patients with articular rheumatism, patients with cancer, and patients with an external wound were applied to the assay of PLA_2M in an undiluted or appropriately diluted form with the assay buffer. The results are shown in Figure 6. As seen from this figure, many of serum samples from patients with articular rheumatism, patients with cancer, and patients with an external wound exhibited a higher level of PLA_2M than that of the serum sample from normal individuals, indicating that the present method is useful for the diagnosis of articular rheumatism and cancers.

Claims

1. A monoclonal antibody recognizing membrane phospholipase A_2.

2. The monoclonal antibody according to claim 1, wherein said membrane phospholipase A_2 is derived from human spleen.

3. The monoclonal antibody according to claim 1, which is monoclonal antibody PL-49, monoclonal antibody PL-71, monoclonal antibody PL-76, or monoclonal antibody PL-78.

4. A hybridoma producing a monoclonal antibody of any one of claims 1 to 3.

5. The hybridoma according to claim 4, which is hybridoma PL-49, hybridoma PL-71, hybridoma PL-76, or hybridoma PL-78.

6. A method for producing a monoclonal antibody of any one of claim 1 to 3, which comprises the steps of:
growing a hybridoma of claim 4 or 5 in the abdominal cavity of a mouse; and
separating said monoclonal antibody from the ascitic fluid accumulated in said abdominal cavity.

7. An immunoassay of membrane phospholipase A_2 using a monoclonal antibody of any one of claims 1 to 3.

8. The immunoassay according to claim 7, which is a radioimmunoassay.

9. The immunoassay according to claim 7, wherein said membrane phospholipase A_2 is sandwiched between two different species of said monoclonal antibodies.

10. The immunoassay according to claim 9, which is an enzyme immunoassay.

Claims for the following Contracting State: ES

1. A method for producing a monoclonal antibody recognizing membrane phospholipase A_2 which comprises the steps of: growing a hybridoma capable of producing said monoclonal antibody in the abdominal cavity of a mouse; and separating said monoclonal antibody from the ascitic fluid accumulated in said abdominal cavity.

2. The method according to claim 1, wherein said membrane phospholipase A_2 is derived from human spleen.

3. The method according to claim 1, wherein the monoclonal antibody produced is monoclonal antibody PL-49, monoclonal antibody PL-71,

monoclonal antibody PL-76, or monoclonal antibody PL-78.

4. A method for preparing a hybridoma cell line producing a monoclonal antibody of any one of claims 1 to 3, which comprises immunizing mice with membrane phospholipase A_2, fusing cells capable of producing antibodies obtained from the immunized mice with myeloma cells and screening the resulting hybridoma cells for production of one of said monoclonal antibodies.

5. The method according to claim 4, wherein the hybridoma prepared is hybridoma PL-49, hybridoma PL-71, hybridoma PL-76, or hybridoma PL-78.

6. An immunoassay of membrane phospholipase A_2 using a monoclonal antibody of any one of claims 1 to 3.

7. The immunoassay according to claim 6, which is a radioimmunoassay.

8. The immunoassay according to claim 6, wherein said membrane phospholipase A_2 is sandwiched between two different species of said monoclonal antibodies.

9. The immunoassay according to claim 8, which is an enzyme immunoassay.

Fig. 1

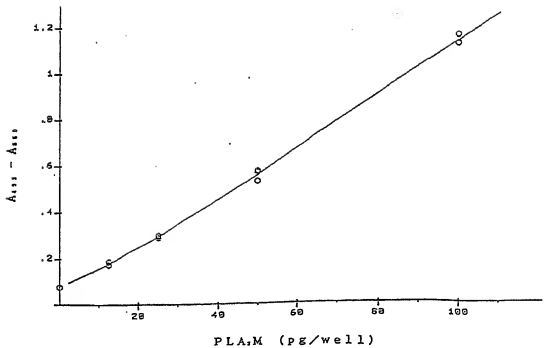
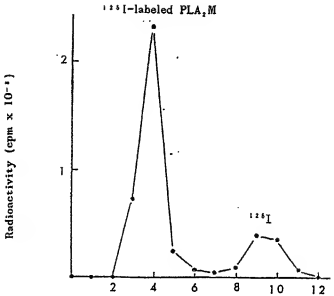
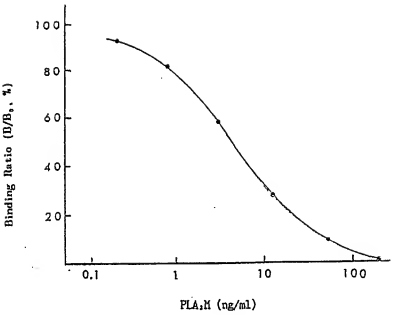


Fig. 2



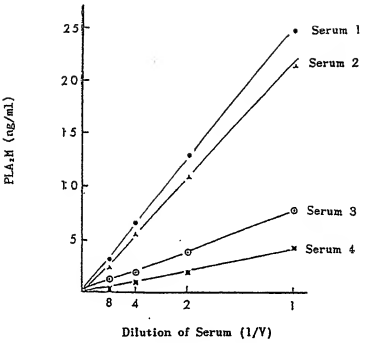
Gel-Filtration of ^{125}I-labeled PLA$_2$M

Fig. 3



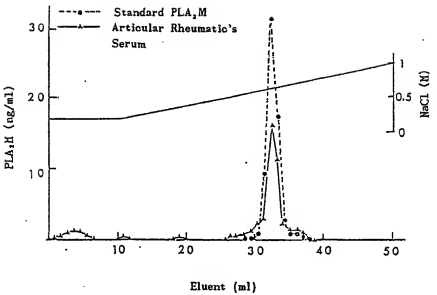
Standard Curve of RIA of PLA_2M

Fig. 4



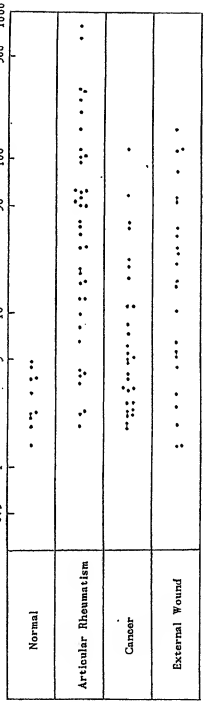
Dilution Curve of Human Serum

Fig. 5



Ion Exchange Chromatogram of
Standard PLA_2M and Articular Rheumatic's Serum

Fig. 6



Serum PLA_2 in Normal Persons and Patients